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PRIORITY DOCUMENT

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Concept House
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South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section74(1) and (4) of the Deregulation and Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the international application filed on 12 MARCH 2002 under the Patent Cooperation Treaty at the UK Receiving Office. The application was allocated the number PCT/GB02/01115

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or the inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

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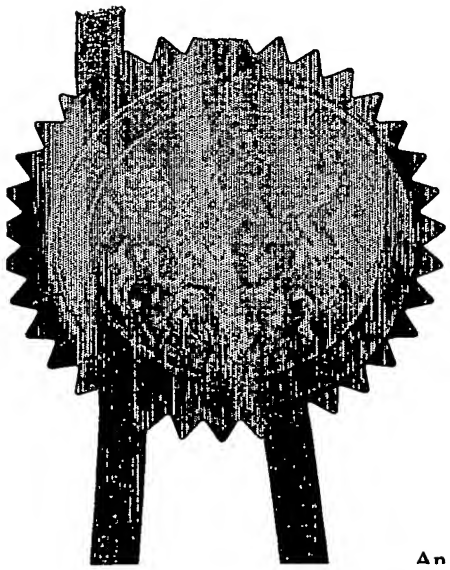

Signed K E Taylor

Date: 25 April 2003

PCT

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REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

PCT/GB 02/01115
International Application No.

12 MARCH 2002 12/03/02
International Filing Date

United Kingdom Patent Office
PCT International Application
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* REP06542WO

Box No. I TITLE OF INVENTION

ENGINEERED BACULOVIRUSES AND THEIR USE

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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State *(that is, country)* of nationality: GB

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This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

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☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

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State *(that is, country)* of residence: FI

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Continuation of Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

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- [ ] applicant only
- [x] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: FI

State *(that is, country)* of residence: FI

This person is applicant for the purposes of:
- [ ] all designated States
- [ ] all designated States except the United States of America
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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

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This person is:
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- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

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- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

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Box No. V DESIGNATION OF STATES *Mark the applicable check-boxes below; at least one must be marked.*

The following designations are hereby made under Rule 4.9(a):

Regional Patent

☒ **AP ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, MZ Mozambique, SD Sudan, SL Sierra Leone, SZ Swaziland, TZ United Republic of Tanzania, UG Uganda, ZM Zambia, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

☒ **EA Eurasian Patent:** AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT

☒ **EP European Patent:** AT Austria, BE Belgium, CH & LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, TR Turkey, and any other State which is a Contracting State of the European Patent Convention and of the PCT

☒ **OA OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GQ Equatorial Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

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☒ AL Albania .....
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☐ ..... ☐ ..... ☐ .....
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Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation (including fees) must reach the receiving Office within the 15-month time limit.)*

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 12.03.01 (12 March 2001)	0106063.1	GB		
item (2)				
item (3)				
item (4)				
item (5)				

☐ Further priority claims are indicated in the Supplemental Box.

The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application was filed with the Office which for the purposes of this international application is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ item (4) ☐ item (5) ☐ other, see Supplemental Box

* *Where the earlier application is an ARIPO application, indicate at least one country party to the Paris Convention for the Protection of Industrial Property or one Member of the World Trade Organization for which that earlier application was filed (Rule 4.10(b)(ii)):* ....

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA / .....

Request to use results of earlier search; reference to that search *(if an earlier search has been carried out by or requested from the International Searching Authority)*:

Date *(day/month/year)* Number Country *(or regional Office)*

Box No. VIII DECLARATIONS

The following declarations are contained in Boxes Nos. VIII (i) to (v) *(mark the applicable check-boxes below and indicate in the right column the number of each type of declaration)*: Number of declarations

☐ Box No. VIII (i)	Declaration as to the identity of the inventor	:
☐ Box No. VIII (ii)	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	:
☐ Box No. VIII (iii)	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	:
☐ Box No. VIII (iv)	Declaration of inventorship (only for the purposes of the designation of the United States of America)	:
☐ Box No. VIII (v)	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	:

Box No. IX CHECK LIST; LANGUAGE OF FILING

This international application contains:

(a) the following number of sheets in paper form:

request (including declaration sheets)	:	5
description (excluding sequence listing part)	:	11
claims	:	2
abstract	:	1
drawings	:	1
Sub-total number of sheets	:	20
sequence listing part of description *(actual number of sheets if filed in paper form, whether or not also filed in computer readable form; see (b) below)*	:	2
Total number of sheets	:	22

(b) sequence listing part of description filed in **computer readable form**

(i) ☐ only (under Section 801(a)(i))

(ii) ☒ in addition to being filed in paper form (under Section 801(a)(ii))

Type and number of carriers (diskette, CD-ROM, CD-R or other) on which the sequence listing part is contained *(additional copies to be indicated under item 9(ii), in right column)*: diskette

This international application is accompanied by the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*:

		Number of items
1. ☒ fee calculation sheet	:	1
2. ☐ original separate power of attorney	:	
3. ☐ original general power of attorney	:	
4. ☒ copy of general power of attorney; reference number, if any:	:	3
5. ☐ statement explaining lack of signature	:	
6. ☐ priority document(s) identified in Box No. VI as item(s):	:	
7. ☐ translation of international application into *(language)*:	:	
8. ☐ separate indications concerning deposited microorganism or other biological material	:	
9. ☐ sequence listing in computer readable form (indicate also type and number of carriers (diskette, CD-ROM, CD-R or other))		
(i) ☐ copy submitted for the purposes of international search under Rule 13*ter* only (and not as part of the international application)	:	
(ii) ☐ *(only where check-box (b)(i) or (b)(ii) is marked in left column)* additional copies including, where applicable, the copy for the purposes of international search under Rule 13*ter*	:	
(iii) ☐ together with relevant statement as to the identity of the copy or copies with the sequence listing part mentioned in left column	:	
10. ☒ other *(specify)*: Patents form 23/77	:	1

Figure of the drawings which should accompany the abstract:

Language of filing of the international application: ENGLISH

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

For The Applicant
Gill Jennings & Every

PERRY, Robert Edward

Date: 12 March 2002

For receiving Office use only

1. Date of actual receipt of the purported international application: 12 MARCH 2002 12/03/02

2. Drawings:
☑ received:
☐ not received:

3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:

4. Date of timely receipt of the required corrections under PCT Article 11(2):

5. International Searching Authority (if two or more are competent): ISA /

6. ☐ Transmittal of search copy delayed until search fee is paid

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See Notes to the request form

ENGINEERED BACULOVIRUSES AND THEIR USE

Field of the Invention

This invention relates to engineered baculoviruses and their use, and especially to libraries and peptide display provided in baculovirus.

Background of the Invention

Over the past few years, many organisms have had their genomes completely sequenced. A draft sequence of the entire human genome has been published. However, sequence information as such does not explain what all the genes do, how cells work, how cells form organisms, what goes wrong in disease, how we age or how to develop a drug. This is where functional genomics, an area of the post-genomic era that deals with the functional analysis of genes and their products, comes into play.

Among the techniques of functional genomics, both DNA microarrays and proteomics hold great promise for the study of complex biological systems. Although DNA microarrays allow high throughput analysis of transcriptome (the complement of mRNAs transcribed from a cell's genome at any one time), genes may be present, they may be mutated, but they are not necessarily transcribed. Some messengers are transcribed but not translated, and the number of mRNA copies does not necessarily reflect the number of functional protein molecules. Proteomics (the complete set of proteins encoded by a cell at any one time) addresses problems that cannot be approached by DNA analysis, namely, relative abundance of the protein product, post-translational modification, subcellular localisation, turnover, interaction with other proteins as well as functional aspects.

The observable characteristics conferred by a gene in an expression library allow the discovery of functional open reading frames in new sequenced genomes (genomic library) as well as the characterisation of function of unknown genes (genomic or cDNA library). A library compatible at the same time with bacterial and eukaryotic cells as well as with *in vitro* and *in vivo* experiments would be a powerful tool in this sense. Although a plasmid vector could allow this in theory, the inefficiency of transduction of eukaryotic cells by plasmid DNA, not to mention the modest gene transfer efficiency of plasmids *in vivo*, decreases the

usefulness of plasmid libraries as high throughput tools of phenomics (automated/ high throughput analysis of proteins).

Baculoviruses have long been used as biopesticides and as tools for efficient recombinant protein production in insect cells. They are generally regarded as safe, due to their naturally high species-specificity and because they are not known to propagate in any non-invertebrate host.

The *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), containing an appropriate eukaryotic promoter, is able to efficiently transfer and express target genes in several mammalian cell types *in vitro*. Further, as reported in WO-A-01/90390, baculoviruses are able to mediate *in vivo* gene transfer comparable to adenoviruses (see also Airenne *et al*, Gene Ther. 7:1499-1504, 2000). The ease of manipulation and rapid construction of recombinant (re-)baculoviruses, the lack of cytotoxicity in mammalian cells, even at a high multiplicity of infection, an inherent incapability to replicate in mammalian cells, and a large capacity (no known insert limit) for the insertion of foreign sequences, are features of baculovirus.

Vp39 is a major capsid protein of baculovirus. Baculovirus enters the cells *via* receptor-mediated endocytosis. The virus is efficiently internalised by many mammalian cell lines, but not able to enter nucleus in non-permissive cells.

It has been previously suggested that the block of an efficient transduction of mammalian cells is not the lack of penetration of the baculovirus into the cells by receptor-mediated endocytosis, but the incapability of the virus to reach the nucleus (Boyce, PNAS USA 93:2348-2352, 1996; Barsoum, Hum. Gene Ther. 8:2011-2018, 1997). There is a general assumption that the block of transduction is in the virus escape from the endosomes.

It is known to engineer the major surface glycoprotein of AcNPV, for the presentation of heterologous proteins on the virus surface (Boublik *et al*, Biotechnology (N.Y.) 13: 1079-1084, 1995). Reference may also be made to O'Reilly *et al*, "Baculovirus expression vectors. A laboratory manual", Oxford University Press, New York, NY (1994).

Summary of the Invention

According to a first aspect of the present invention, a method for selecting a target gene, comprises the steps of:

(i) generating a library of genes or genomic fragments cloned in baculovirus as a vector;

(ii) transforming a host cell with the vector; and

(iii) detecting gene expression under predetermined conditions.

Baculoviral genomic or cDNA libraries offer a powerful tool for phenomics, by enabling the functional screening of the constructed libraries in eukaryotic cells both *in vitro* and *in vivo*. Addition of a bacterial promoter into a baculovirus donor vector will also allow expression screening of cDNA libraries in bacterial cells. Baculovirus libraries may be constructed from suitable validated full-length clones and sequences from human and other vertebrate sources. This will allow integration of the efficient infection (insect cells) and transduction (vertebrate cells) of target cells by baculoviruses, and application to phenomics.

According to a second aspect of the invention, the baculovirus capsid is modified to display one or more heterologous proteins. In particular, it has now been shown that the major block in baculovirus transduction of mammalian cells is not in endosome escape, but in nuclear transport of the virus capsid.

It has also been shown that new protein entities can be fused to the N- or C-terminus of vp39 without compromising the viral titre and functionality of the vp39 fusion proteins on the AcMNPV capsid surface. Furthermore, the tagged virus can be used for gene transfer *in vivo*. The constructed baculovirus thus provides a versatile tool for real-time analysis of the transduction route of AcMNPV in mammalian cells and intact animals as well as infection mechanism in insect cells. Capsid-modified baculoviruses also hold a great promise for the nuclear and subcellular targeting of transgenes and as a new peptide display system for eukaryotic cells.

The capsid display system has many advantages compared to a gp64 envelope display system. In vp39, no structural motifs have been recognised either for association with molecules within the stromal matter or for capsid

assembly, nor is it responsible for infectivity of the virus. In addition, immunoelectron microscopy shows that vp39 is randomly distributed on the surface of the capsid as opposed to gp64 on the virus envelope. Baculovirus envelope display system allows only fusions to N-terminal end of the gp64, whereas vp39 allows tagging to both terminus. Although it remains to be shown how large proteins can be, displayed on the baculovirus capsid, results suggest that at least 27 kDa protein can be efficiently expressed. Because the length of the capsid can extend relatively freely, it is reasonable to expect that vp39 is also compatible with larger proteins, e.g. up to 100 kDa or higher. Random display of peptides or proteins on the capsid may allow the discovery of moieties capable of transporting the capsid into the nucleus or other intracellular organels.

Further uses for modified baculovirus according to the invention include any form of "capsid therapy". Thus, proteins can be used as a system for the transport of peptides or proteins directly into the nucleus.

Description of the Invention

In order to direct a high level expression of baculovirus library genes in invertebrate, *E. coli*, and insect cells, an expression cassette may be constructed, based on a hybrid or other suitable promoter which allows high level expression of target genes both in prokaryotic and eukaryotic cells. Target site for cre-recombinase (*loxP*) may be included into the expression cassette, to allow easy construction of baculovirus libraries using site-specific recombination *in vitro* (Sauer, Methods 14:381-392, 1998). To further increase the options to construct the baculovirus libraries, attR and ccdB sites (and, say, a chloramphenicol-resistance or other marker to select for successful ligation of the cassette) can be included into expression cassette. This enables facile conversion of Life Technologies Gibco BRL® Gateway™ Cloning Technology (Life Technologies) compatible libraries to the novel baculovirus library. In addition to cre/lox and Gateway compatibility, the expression cassette can allow traditional library construction by several unique restriction enzymes available in vector MCS after modifications such as those described above.

The constructed expression cassette may be cloned into any suitable baculovirus plasmid or baculovirus system which can act as a donor vector. pFastBac-1 is a preferred backbone plasmid since it is compatible with Bac-To-Bac™ baculovirus expression system (Gibco BRL) which allows rapid and easy preparation of re-baculoviruses by site-specific transposition in *Escherichia coli*. If desired, the cassette can also be integrated to any desired plasmid/expression system, e.g. into a version of Bac-TO-Bac™ baculovirus expression system that permits more efficient and direct construction of baculoviruses (Leusch *et al*, Gene 160:191-194, 1995).

The expression cassette can also be cloned as part of the baculovirus genome and library construction then performed directly to it by cre/lox, Gateway or direct cloning methods.

All cloning work can be performed using standard molecular biology methods. Constructed baculovirus libraries will be screened for expression/phenotype effect(s) in suitable *E. coli* strain(s) (library in donor plasmid format), insect cells and vertebrate cells. Selected viruses or whole libraries can also be used directly for *in vivo* studies. This alleviates the great and unique potential of the new baculovirus libraries; the same library can be used for prokaryotic and eukaryotic cells and in cell (*in vitro*) and animal (*in vivo*) studies.

By way of example, and in order to allow intracellular targeting of AcMNPV, a baculovirus capsid display system has been developed. The system is based on a versatile donor vector which allows efficient production of desired proteins as N- or C-terminal fusion to the baculovirus major capsid protein, vp39 (Thiem & Miller, J. Virol. 63:2008-2018, 1989). Alternative baculovirus capsid proteins which are potential targets for peptides or proteins include p24 and p80.

A construct of high titre re-AcMNPV can display a high concentration of a foreign protein in its capsid. The tagged virus is a facile tool to study the route of baculovirus transduction in mammalian cells from the cell surface into the nucleus and transfection capacity of baculovirus *in vivo*. The system provides at the same time a powerful tool to study the bottlenecks of AcMNPV transduction of non-permissible cell lines and a possibility to improve nuclear or

subcellular targeting by incorporation of specific sequences in vp39 protein. AcMNPV may also allow double-targeting at the cell surface level by insertion of specific ligands or antibodies to the envelope, followed by intracellular targeting by vp39 modification.

To maximise the chance to achieve a functional fusion and capsid assembly, a transfer plasmid was constructed which enables fusion of desired entities either into N- or C-terminus of the vp39 (Fig. 1). Fusion protein production is driven by a strong polyhedrin promoter, e.g. as disclosed by O'Reilly *et al*, *supra*. Since computer prediction showed that vp39 had low complexity at C-terminus but was constrained at N-terminus, a linker sequence (e.g. GGGGS) may be added to the N-terminus, to give distance and flexibility for N-terminal fusion proteins to fold correctly. An option to tag the vp39 fusion proteins with a His-tag may also be preferred. For example, the pBACcap-1 plasmid produces vp39 with His-tag at the N-terminus. However, the same transfer plasmid can be used for N- or C-terminal fusions with or without His-tag. The system is compatible with transporon-mediated virus preparation. However, the expression cassette in the pBACcap-1 can be easily moved to any desired baculovirus vector.

The present invention includes the possibility of double-targeting, as an extension of the conventional targeting working primarily at tissue or cell surface level. The basic idea of the tissue targeting is to add a specific ligand on the surface of the gene transfer vector to achieve specific binding to desired cells or tissues. It is well known that a specific ligand-receptor interaction does not guarantee efficient transduction of the target cell. Internalisation, escape from endosomes and transport of the genetic material into nucleus are also required. Although the transduction can be improved by selection of cell membrane targeting moieties, the route from cytosol to nucleus remains difficult to achieve. Enveloped viruses hold a promise for an efficient double-targeting at the tissue and intracellular levels. By modifying the envelope with a desired tissue targeting moiety and the capsid with an intracellular targeting moiety, efficient and specific transduction of the target cells should be achieved. Transcriptional targeting with specific promoters may also be added to these vectors.

The following Examples illustrate the invention.

Methods

Capsid Display Vector

In order to construct a general baculovirus vector for capsid display, the region corresponding to nucleotides (nt) 469-1506 of vp 39 (Genbank:M22978) was amplified from the purified bacmid DNA (Luckow *et al*, J. Virol. 67, 4566-4579, 1993) by polymerase chain reaction (PCR). The forward primer was 5' - TT GAA AGA TCT GAA TTC *ATG CAC CAC CAT CAC* CAT CAC GGA TCC GGC GGC GGC GGC TCG **GCG GCT AGT GCC CGT GGG T** - 3' (specific sequence for nt 469-486 of vp39 gene in bold; *Bgl*II, *Eco*RI, *Bam*HI, sites underlined; 6 X Histidine tag with start codon in italics); the reverse primer was 5' -TT CTG GGT ACC GCt tta *ATG GTG ATG ATG GTG GTG* TCT AGA GCt tta ACT AGT **GAC GGC TAT TCC TCC ACC** - 3' (specific sequence for nt 1489-1506 of vp39 gene in bold; *Kpn*I, *Xba*I and *Spe*I sites underlined; 6 X Histidine tag in italics; stop codon in small caps). The PCR was performed essentially as described by Airenne *et al*, Gene 144:75-80, 1994, except annealing was set to 58°C. Amplified fragment was digested with *Bgl*II and *Kpn*I enzymes and purified as described in Airenne *et al*, *supra*. The purified PCR product was cloned into *Bam*HI+*Kpn*I -digested pFastBAC1 vector (Invitrogen, Carlsbad, USA). The resulted plasmid was named as pBACcap-1. The nucleotide sequence was confirmed by sequencing (ALF; Amersham Pharmacia Biotech, Uppsala, Sweden).

Preparation of EGFP Displaying Viruses

cDNA encoding EGFP (enhanced green fluorescent protein) was amplified from the pEGFP-N1 plasmid (Genbank:U55762, Clontech, Palo Alto, USA) by PCR and cloned into the pBACcap-1. Two sets of primers were used to enable EGFP fusion both to N- and C-terminal ends of the vp39. For the N-terminal fusion, the forward primer was 5' - CGG GAT *GAA TTC* **GTC GCC ACC ATG GTG AGC AAG GGC GAG GAG** - 3' (specific sequence for nt 670-699 of pEGFP-N1 in bold; *Eco*RI site in italics), and the reverse primer 5' - GCG GCC *GGA TCC* **CTT GTA CAG CTC GTC CAT GCC** - 3' (specific sequence for

nt 1375-1395 of pEGFP-N1 in bold; *Bam*HI site in italics). The amplified fragment which corresponded to nt 670-1395 of pEGFP-N1 was cloned into *Eco*RI/*Bam*HI site of the *Spe*I/*Xba*I deleted pBACcap-1. The resulting plasmid was named EGFPvp39.

For the C-terminal version, the forward primer was 5' - GTC GCC *ACT AGT* **GTG AGC AAG GGC GAG GAG CTG** -3' (specific sequence for nt 682-702 of pEGFP-N1 in bold; *Spe*I site in italics), and the reverse primer 5' - AGA GTC *ACT AGT* GCt **tta CTT GTA CAG CTC GTC CAT GCC** - 3' (specific sequence for nt 1375-1398 of pEGFP-N1 in bold; *Spe*I site in italics; stop codon in small caps). The amplified fragment which corresponded to nt 682-1398 of pEGFP-N1 was cloned into *Spe*I site of the pBACcap-1. The resulting plasmid was named vp39EGFP. The nucleotide sequences were confirmed by sequencing (ALF).

Viruses were generated using Bac-To-Bac system™ according to manufacturer's instructions (Invitrogen). Viruses were concentrated and gradient-purified, as described by Airenne *et al*, Gene Ther. 7:1499-1504, 2000. Virus titre was determined by end-point dilution assay on Sf9 cells. Sterility test was performed for virus preparations and they were analysed to be free of lipopolysaccharide and mycoplasma contamination.

Immunoblotting

Samples corresponding to about 60000 infected cells or virus from 4 ml of culture medium were loaded onto 10% SDS-PAGE under reducing conditions. The gel was blotted onto nitrocellulose filter and immunostained as described by Airenne *et al* (1994), *supra*. Polyclonal rabbit anti-EGFP (Molecular Probes Inc., Eugene, USA) was used as a primary antibody (1:4000) and goat anti-rabbit serum as a secondary antibody (1:2000) (Bio-Rad, Hercules, USA). Molecular weight standard in the SDS-PAGE was from Bio-Rad.

Electron Microscopy

For immunoelectron microscopy, vp39EGFP baculovirus particles were bound to formwar-coated metal grids treated with 5% foetal calf serum in PBS. Grids were then treated with protein A gold for 25 min (5 nm in diameter, G.

Posthuma and J. Slot, Utrecht, The Netherlands) and washed with PBS for 25 min. The grid was fixed with 2.5% glutaraldehyde and contrasted and embedded using 0.3% uranyl acetate in 1.5% methyl cellulose. The HepG2 and EAHY cells infected with the virus were fixed with 2.5% glutaraldehyde for 1 h at room temperature and then with 1% osmium tetroxide for 1 h at +4°C. After dehydration, cells were stained with 2% uranyl acetate for 30 min at room temperature, embedded in Epon and sectioned for electron microscopy. Sections were stained with lead citrate and uranyl acetate. Samples were examined using JEM-1200 EX electron microscope (Jeol Ltd., Tokyo, Japan).

Immunofluorescence and Confocal Microscopy

Subconfluent human hepatoma cell line HepG2 and human endothelial aortic hybridoma (EaHy926, Dr. Edgell, University of North Carolina, USA) cultures were infected by vp39EGFP baculovirus as follows: cells were first washed with PBS on ice, the virus was added on the cells in DMEM containing 1% foetal calf serum using multiplicity of transduction of 80-100 and incubated for 1 h on ice. Cells were washed with PBS containing 0.5% BSA. Then, complete DMEM (containing 10% serum) was added and cells were incubated for various time periods at 37°C and finally fixed with 4% paraformaldehyde in PBS for 20 min. Cells were labelled with early endosome antigen 1 (BD Transduction Laboratories, Lexington, Kentucky). Goat secondary antibodies against mouse antibodies (Alexa red 546 nm; Molecular Probes Inc., Eugene, Oregon) were used in the labelling. The cells were mounted in mowiol and examined with an Axiovert 100 M SP epifluorescence microscope (Carl Zeiss, Jena, Germany) and a confocal microscope (Zeiss LSM510). For visualising EGFP and Alexa red 546, multitracking for 488 and 546 laser lines was used in order to avoid false co-localisation. Live confocal microscopy on HepG2 and EAHY cells was performed as follows: cells were plated on chambered coverglasses (Nalge NUNC, Naperville, Illinois). After virus binding on ice, cells were transferred to confocal microscope with a heated working stage and objective controlled by Tempcontrol 37-2 (Carl Zeiss, Jena, Germany). Cells that were positive for EGFP were scanned with various time intervals using the programme in LSM 510 software (version 2.3; Carl Zeiss, Jena, Germany).

In vivo Injection into Rat Brain

Male Wistar rats (320-350 g) were anaesthetised intraperitoneally with a solution (0.150 ml/100 g) containing fentanyl-fluanisone (Janssen-Cilag, Hypnorm®, Buckinghamshire, UK) and midazolame (Roche, Dormicum®, Espoo, Finland) and placed into stereotaxic apparatus (Kopf Instruments). A burr hole was done into the following stereotaxic coordinates: 1mm to the midline and +1 mm to bregma. 100 µl of the EGFPvp39 or vp39EGDP baculoviruses (0.9×10^{10} pfu/ml) in 0.9 N NaCl was injected during 4 x 5 min periods using a Hamilton syringe with a 27-gauge needle to a depth of 3.5 mm. Animals were sacrificed with CO_2 7 h after the gene transfer. Rats were perfused with PBS by the transcardiac route for 10 min, followed by fixation with 4% paraformaldehyde/0.15 M sodium-phosphate buffer (pH 7.4) for 10 min. Brains were removed, snap-frozen with isopenthane and 40 µm thick frozen sections were prepared. Slides were immediately analysed with fluorescence microscopy (Olympus AX70 microscope, Olympus Optical, Japan) and data were collected with Image-Pro Plus software.

Results

Characterisation of EGFP Displaying Viruses

Sf9 cells infected with EGFPvp39 or vp39EGFP encoding viruses produced expected 67 kDa bands in immunoblots. The same results were obtained from purified virus preparations. The results suggested that both vp39 variants were efficiently produced in insect cells and incorporated into virus particles. However, to confirm that the fusion proteins were part of the virus capsids, vp39EGFP virus was incubated with anti-EGFP followed by immunogold labelling and examined by immunoelectron microscopy. Viruses showed a typical rod-shaped morphology surrounded by a characteristically loose-fitting envelope and the surfaces of the unenveloped capsids were heavily gold-labelled. The results confirmed that EGFP was evenly displayed on the surface of the recombinant baculovirus capsid.

In order to get an estimate of the amount of the incorporated vp39EGFP per virus particle, serial dilutions of the purified virus particles were immunoblotted and compared to the known amount of the purified EGFP.

According to the analyses, 431-862 molecules of the vp39EGFP were incorporated per virus particle. Similarly, 585 vp39EGFP molecules per capsid was measured by comparing the detected fluorescence of the vp39EGFP virus preparation to EGFP control. The high incorporation rate was also supported by Coomassie-stained SDS-PAGE, according to which a high proportion of the capsid was made of the vp39EGFP and by easy visualisation of the virus preparations by confocal microscopy. Assembly of the viruses was not affected by the fusion protein, since the titres of the EGFPvp39 and vp39EGFP viruses were 9.5×10^9 and 8.8×10^9 pfu/ml, respectively.

Baculovirus-Mediated Transduction

The intracellular route of vp39EGFP virus was followed by monitoring EGFP-tagged capsids and fluorescently labelled cellular compartments by confocal microscopy. Both EAHY and HepG2 cells were transduced for various time periods and the co-localisation of the virus with an early endosome antigen 1 (EEA1) was studied.

In HepG2 cells, the virus particles were present in nuclei 4 h p.t., shpowing that intact capsids were translocated to the nucleus after the release from the early endosomes. Electron microscopy of EAHY cells confirmed that no virus particles were present in the nuclei 4 h p.t.

Visualisation in Rat Brain

The virus was still clearly seen 7 h after *in vivo* injections into the right corpus callosum of rat brain near the injection site. Thus, vp39EGFP baculovirus can be used for immediate analysis of virus localisation *in vivo*.

CLAIMS

1. A method for selecting a target gene, which comprises the steps of:
   (i) generating a library of genes or genomic fragments cloned in baculovirus as a vector;
   (ii) transforming a host cell with the vector; and
   (iii) detecting gene expression under predetermined conditions.

2. A method according to claim 1, wherein the predetermined conditions comprise a set of different conditions under which expression of the target gene may or may not be detected.

3. A method according to claim 2, wherein the different conditions comprise limiting dilution.

4. A method according to any preceding claim, wherein step (iii) comprises identification of a phenotype.

5. A method according to any preceding claim, wherein step (iii) is repeated following selection of one or some of the products of the predetermined conditions.

6. A method according to any preceding claim, which additionally comprises characterising the gene expressed under the predetermined conditions.

7. A method according to any preceding claim, wherein the genes are human genes.

8. A method according to any preceding claim, wherein the baculovirus vector contains at least 3 genes.

9. A method according to any preceding claim, wherein each gene is at least 10 kb long.

10. A method according to any preceding claim, wherein the host cell is an insect cell.

11. A method according to any of claims 1 to 9, wherein the host cell is a mammalian cell.

12. Baculovirus whose capsid has been modified to display one or more heterologous peptides or protein.

13. Baculovirus according to claim 12, wherein vp39, p24 or p80 is modified.

14. Baculovirus according to claim 13, wherein vp39 is modified with a fusion protein at the N- and/or C-terminus.

15. Baculovirus according to any of claims 12 to 14, wherein the modification allows nuclear or subcellular targeting.

16. Use of baculovirus according to any of claims 12 to 15, for the delivery of a peptide or protein into the nucleus.

ABSTRACT

ENGINEERED BACULOVIRUSES AND THEIR USE

A method for selecting a target gene, comprises the steps of:

(i) generating a library of genes or genomic fragments cloned in baculovirus as a vector;

(ii) transforming a host cell with the vector; and

(iii) detecting gene expression under predetermined conditions.

Baculovirus whose capsid has been modified to display one or more heterologous peptides or protein is a further aspect.

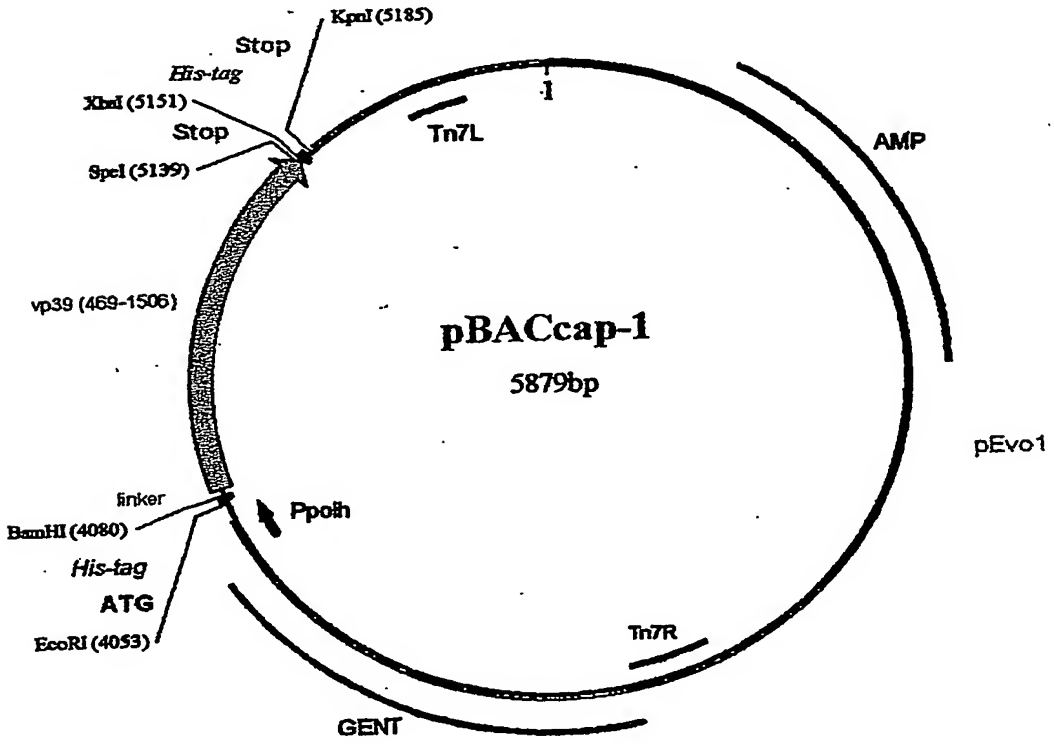
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FIG. 1

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